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Novel enzyme immunoassay for thyrotropin-releasing hormone using *N*-(4-diazophenyl)maleimide as a coupling agent

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A novel enzyme immunoassay (EIA) for thyrotropin-releasing hormone (TRH) was developed which used *N*-(4-diazophenyl)maleimide (DPM) as a new heterobifunctional agent capable of cross-linking TRH to mercaptosuccinyl bovine serum albumin and to β-D-galactosidase. The resulting conjugates act as the immunogen producing anti-TRH serum in rabbits and the enzyme marker of TRH in the EIA, respectively. This EIA with a double-antibody technique was sensitive and reproducible in measuring TRH at concentrations as low as 50 pg per tube, and monospecific to the hormone showing no cross-reactivity with the hormone analogue L-pGlu-L-His-L-Pro and TRH constituents. Using this assay, the distribution of immunoreactive TRH in the brain was determined easily in rats. The use of DPM should provide a valuable new method for developing EIA hitherto possible for other peptide hormones containing neither a free carboxy nor a free amino group, using imidazole, phenolic, and indole group(s) of the amino acid as a reaction site.

Thyrotropin-releasing hormone *Enzyme immunoassay* N-*(4-Diazophenyl)maleimide*
Heterobifunctional cross-linking agent β-*D-Galactosidase*

1. INTRODUCTION

Thyrotropin-releasing hormone (TRH) not only stimulates thyrotropin (TSH) release from the adenohypophysis but is also known to exert a variety of biological effects on prolactin secretion and the central nervous system.

Among the various methods of detection of TRH, radioimmunoassay (RIA) has been most widely employed [1–6]. Recent studies have shown that enzyme immunoassay (EIA) overcomes drawbacks inherent in radiolabeling and may have a sensitivity comparable to RIA [7,8]. However, an EIA system for TRH has not previously been developed because of the lack of suitable methods for preparing a homogeneous conjugate of an enzyme and TRH (which contains neither a free carboxyl nor a free amino group) that could be utilizable as a tracer.

This report describes the use of *N*-(4-diazophenyl)maleimide (DPM) as a novel heterobifunctional agent cross-linking TRH through its imidazole group of histidine with thiol groups of mercaptosuccinylated bovine serum albumin and of β-D-galactosidase (EC 3.2.1.23). The resultant conjugates were used respectively as the immunogen to TRH and enzyme marker of TRH in developing EIA for TRH.

2. MATERIALS AND METHODS

2.1. *Materials*

Synthetic TRH (L-pyroglu-L-His-L-ProNH$_2$) and L-pyroglu-L-His-L-Pro were purchased from the Protein Research Foundation (Mino, Osaka) and Bioproducts (Brussels, Belgium), respectively. *N*-(4-Aminophenyl)maleimide (APM) was bought from Tokyo Kasei Kogyo (Tokyo) and β-D-galactosidase from *Escherichia coli* from Boehringer-Mannheim (Mannheim, FRG).

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2.2. *Preparation of immunogen to TRH*

The reactions involved in the preparative process used in this stsudy are shown in fig.1.

2.3. *Introduction of maleimide group into TRH*

DPM was prepared by dissolving 1.7 mg (9.2 μmol) APM in 175 μl of 1 N acetic acid and adding 1.3 mg (18.8 μmol) of sodium nitrite in 100 μl distilled water. The diazotization reaction was allowed to continue for 10 min at 4°C with intermittent stirring. This solution was added to 3.0 mg (8.27 μmol) TRH in 1.5 ml of a mixed solution of 1 M 2-amino-2-methyl-1,3-propanediol·HCl buffer (pH 8.7) and tetrahydrofuran (1:1, v/v) and the mixture incubated for 10 min at room temperature with stirring. Ethyl acetate (1.0 ml) was then added, and the mixture shaken vigorously followed by centrifugation at 1000 rpm. The aqueous layer (TRH-DPM) was used without further purification for preparing the conjugates with mercaptosuccinyl bovine serum albumin (MS·BSA) and with β-galactosidase as the TRH immunogen and the tracer in the EIA, respectively.

2.4. *Immunogen to TRH*

TRH-DPM (~4.49 mg, 8.0 μmol) was incubated with MS·BSA containing 17 thiol groups per mol (5 mg) [7,8] in 1.0 ml of 0.1 M phosphate buffer (pH 7.0) containing 3 M urea at room temperature for 30 min with stirring. The conjugate (TRH-

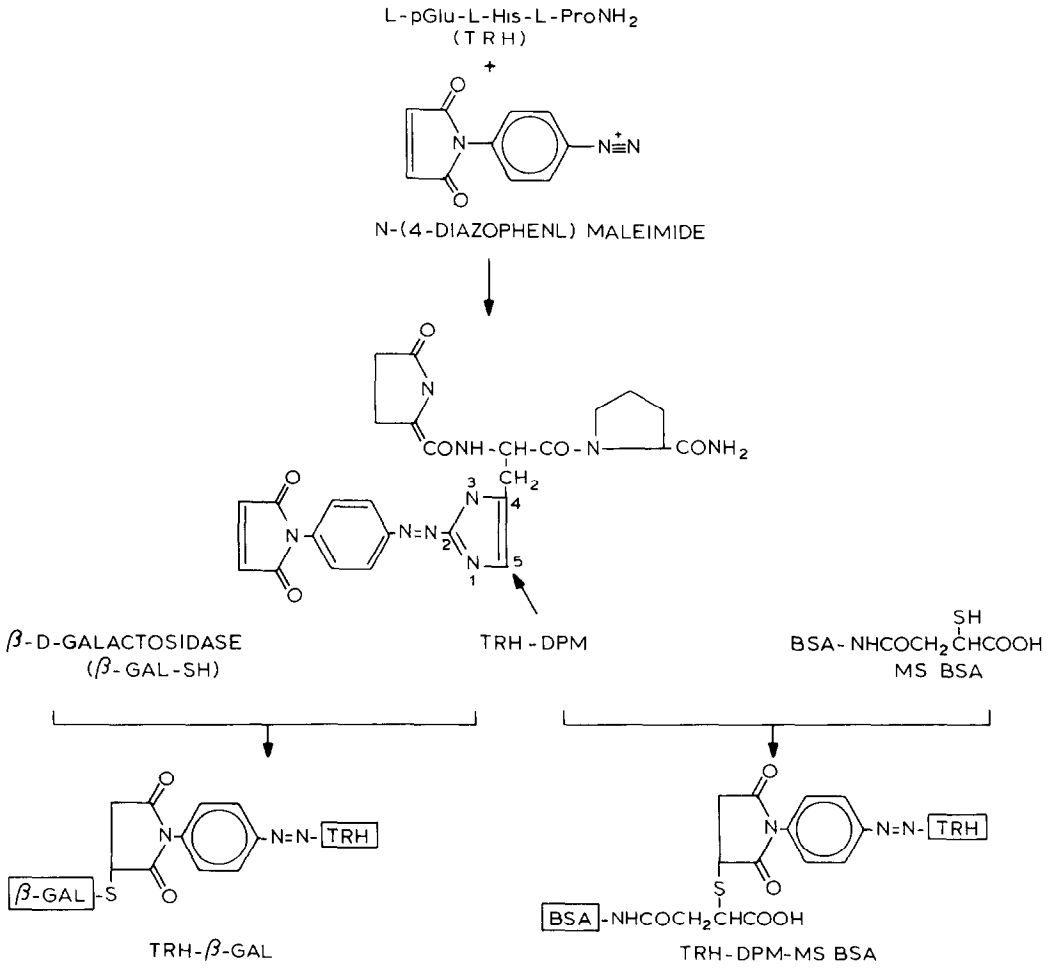

Fig.1. Scheme for preparation of the immunogen for TRH and of TRH-β-galactosidase conjugate. β-GAL, β-galactosidase.

DPM-MS·BSA) was purified by column chromatography on Sephadex G-100 (2.8 × 42 cm) with an eluent of 0.1 M phosphate buffer (pH 7.0) containing 3 M urea.

2.5. *Immunization*

Three female rabbits were given s.c. and i.m. injections of approx. 1.0 mg TRH-DPM-MS·BSA emulsified in Freund's complete adjuvant. Booster injections were then given 4 times at biweekly intervals, using one-half the amount of the dose of the first immunization.

2.6. *Enzyme labeling of TRH (fig.1)*

TRH-DPM (~38.1 μg, 0.1 μmol) was incubated with β-galactosidase (39 μg, 0.07 nmol) [9] in 1.0 ml of 0.1 M phosphate buffer (pH 6.0) at room temperature for 30 min. The reaction mixture was chromatographed on a column of Sephadex 6B (2 × 38 cm) with 20 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl, 0.1% BSA and 0.1% NaN_3 and with 3-ml fractions. Fractions of the main peak of the pure enzyme activity were chosen as a label in the EIA.

2.7. *Distribution of immunoreactive TRH in the brain*

Six Wistar rats weighing about 250–300 g were killed by decapitation. The brain was chilled quickly in cold 0.15 M NaCl and dissected into the cerebrum, thalamus, hypothalamus, brain stem and cerebellum as described by Oliver et al. [10]. Each tissue was weighed and homogenized in 5 ml of 90% methanol (v/v) using a Polytron Model PT 10 (Brinkman Instruments, Westbury, NY). The homogenates were centrifuged at 0°C for 15 min at 2500 rpm and the supernatants collected and evaporated to dryness in vacuo. The residue was dissolved in 0.06 M sodium phosphate, pH 7.4, containing 0.01 M EDTA, 0.1% BSA and 0.1% NaN_3 and measured by EIA.

2.8. *Measurement of β-galactosidase activity and double-antibody EIA method*

These were performed essentially as described [7,8,11].

3. RESULTS

3.1. *Antibody response*

TRH antisera were produced following the priming injection in each of the 3 rabbits immunized. Subsequent injections considerably increased the antiserum titers in the rabbits. The titers in the serum samples were measured in terms of enzyme activity of TRH-β-galactosidase bound to solutions of antiserum obtained by the EIA. Antiserum used in this study was obtained 2 weeks after the final injection, with the highest titer of the antibody approx. 20% (B/T) of the total enzyme activity at final dilution 1:3000.

3.2. *EIA of TRH*

A standard calibration curve of TRH measurement is shown in fig.2. The sensitivity of TRH assays, defined as the smallest amount distinguishable from zero at the 95% confidence level, is 50 pg/tube. TRH analogue (pGlu-His-Pro) and its constituents (pGlu-His, $ProNH_2$, pGlu, His, and Pro) are tested for their ability to displace bound TRH-β-galactosidase conjugate, demonstrating that none compete significantly with the tracer against anti-TRH antibody in the EIA.

The EIA measured the immunoreactive TRH in various brain tissues of male rats (table 1). The concentration was at its highest level in the hypothalamus (310 ng per g tissue), followed by 55, 20, 5.5 and 2.6 ng/g in the thalamus, brain stem, cerebrum and cerebellum, respectively.

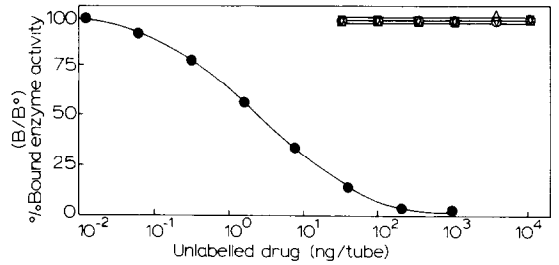

Fig.2. Standard curve for TRH, and cross-reactivity of TRH analogue and TRH constituents with anti-TRH serum. The curves show the amount (%) of bound enzyme activity for various doses of TRH and TRH-related compounds (*B*) as a ratio of that bound using TRH-β-galactosidase alone (B^0). (●) TRH, (○) L-pGlu-L-His-L-Pro, (△) pGlu-His, (▽) pGlu, (□) His, (◯) $ProNH_2$, (◑) Pro.

Table 1

Endogenous immunoreactive TRH contents in various brain tissues in rats

Tissue	Tissue wet wt (mg)	TRH content (ng)	TRH concentration (ng/g tissue)
Hypothalamus	20.6 ± 2.0	6.39 ± 0.42	310 ± 25
Thalamus	256.0 ± 10.5	14.30 ± 2.05	55 ± 3.0
Brain stem	212.8 ± 12.0	4.35 ± 0.45	20 ± 1.3
Cerebrum	1250.0 ± 23.0	6.90 ± 0.89	5.5 ± 0.32
Cerebellum	260.0 ± 8.2	0.68 ± 0.02	2.6 ± 0.10

Each value is the mean ± SE obtained from 6 rats

4. DISCUSSION

Since 1972, a number of RIAs for the measurement of TRH have appeared [1–6]. The TRH immunogen has been prepared by coupling TRH to carrier proteins by the use of the cross-linking agents bis-diazotized benzidine [1–3], *p*-diazoniumphenylacetic acid [14] or 1,5-difluoro-2,4-dinitrobenzene [5], or directly coupling a TRH analogue, pGlu-His-Pro to the amino groups of a protein carrier with a carbodiimide [8]. These cross-linking agents, however, are unsuitable for preparing TRH-enzyme conjugate for use in developing EIA as a tracer, because these methods might produce as side-products complex polymers of enzyme and TRH.

We used DPM (prepared by the diazotization reaction of the commercially available APM) as a heterobifunctional agent to conjugate TRH with proteins (MS·BSA or β-galactosidase) by means of its 2 selective functional groups of diazo and maleimide acting on the imidazole of the histidyl moiety of TRH and the thiol groups of the proteins, respectively, in a two-step process. The first azo-coupling may occur at both or either of the points C-2 and C-5 in the imidazole group (see fig.1). The maleimide group of DPM was, however, unstable during the azo-coupling reaction with TRH at a weakly alkaline pH, and the conditions described in section 2 which preclude hydrolysis of the agent were chosen. Following the removal of unreacted APM by extraction with ethyl acetate, the maleimide group incorporated on a TRH molecule was then conjugated by thiolation with the thiol groups of MS·BSA and β-galactosidase, and the resulting conjugates were used respectively as the immunogen producing anti-TRH serum and the enzyme marker of TRH in the EIA. The present method based on a two-step process is very mild and does not result in extensive (intra- or intermolecular) self-coupling of TRH or proteins as occurs when other commonly used coupling agents are employed [1–6]. The TRH-DPM-MS·BSA thus prepared elicited the production of anti-TRH antibody in all 3 rabbits immunized, with the highest titer 2 weeks after the final booster injection. TRH-β-galactosidase as a tracer retained full enzyme activity of β-galactosidase without loss during the conjugation reaction, and showed reasonable stability, remaining unchanged in its immunoreactive enzyme activity when stored at 4°C for several months. These reagents were used to develop an EIA for TRH which proved sensitive (with TRH at 50 pg/tube, almost comparable sensitivity to RIA can be measured) and reproducible (10.8% variation interassay as an average, not shown). This assay was quite specific to TRH and showed no cross-reactivity with TRH analogue and TRH constituents at a high concentration of 10 μg/tube. The utility of the EIA was demonstrated by its application to measure the brain distribution of immunoreactive TRH in rats (table 1). The TRH levels in the tissues tested were very slightly higher and almost comparable to those reported by Oliver et al. [11] by means of RIA for TRH.

Based on the results of the present study, it is expected that DPM could serve as a potent useful

heterobifunctional agent capable of cross-linking many other peptide hormones (even those which contain neither a free carboxyl nor a free amino group) to the thiol groups of proteins through imidazole, phenolic, indole and a variety of other groups of the amino acid. The resulting conjugates may be used as the immunogen and the enzyme marker of the hormones in the EIA.

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